Quantitative Assessment of Hemadsorption by Myxoviruses: Virus Hemadsorption Assay

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The standardization and quantitative evaluation of an assay for myxoviruses, based on the enumeration of individual infected clone 1-5C-4 cells manifesting hemadsorption within 24 h of infection, are described. Hemadsorption was detectable earlier than immunofluorescence in infected cells or hemagglutinins in culture medium. The relationship between virus concentration and cells exhibiting hemadsorption was linear. The assay was highly precise, sensitive, and reproducible.

The phenomenon of hemadsorption, first described by Vogel and Shelokov (15), with influenza viruses grown in cell cultures subsequently proved cardinal to the discovery and serological characterization of the parainfluenza viruses (1). The applicability of the phenomenon for assaying myxoviruses, however, has been based on techniques that either lacked simplicity of performance, adequate quantitative evaluation, or, relying on multiple cycles of virus propagation in host cells, required prolonged incubation periods. Some hemadsorption assay procedures that have been employed with myxoviruses include counting clumps of agglutinated erythrocytes in 48-h infected-cell cultures (13), the virus dilution end-point method of hemadsorption which requires incubation of infected cell cultures for 5 days (9), and a 48-h hemadsorption plaque assay (3). An attempt was made to develop a virus hemadsorption assay, similar in principle to immunofluorescent cell-counting virus assays (5, 6), that is rapid, quantitative, and dependent on a single cycle of cell infection.

This report describes the application and standardization of a quantitative assay for myxoviruses based on the enumeration of individual infected cells manifesting hemadsorption within 24 h of infection.

MATERIALS AND METHODS

Cell culture. The principal cell line used was clone 1-5C-4 derived from a variant line of Chang's conjunctival cell (16). Cells were propagated with Eagle minimum essential medium containing 10% fetal calf serum and maintained with minimal essential medium plus 5% fetal calf serum. For the virus hemadsorption assay, cells were cultivated on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (19 by 65 mm). One milliliter of cell suspension, containing approximately 10^5 cells, was introduced onto each cover slip which was then incubated at 35 C for 24 h or until a complete cell monolayer was formed.

Virus. Influenza A₀/PR8 and parainfluenza 3 C-243 virus strains employed in this study were obtained from the American Type Culture Collection, Rockville, Md. Influenza virus was grown in the allantois of 11-day-old chicken embryonated eggs. After incubation at 35 C for 48 h, eggs were chilled (6 C) for 5 or more h. Allantoic fluids were then harvested aseptically and individually assayed for hemagglutinin (HA), and those with the highest HA titer were pooled. The stock virus preparation was stored in 1-ml amounts in glass vials at −70 C. To propagate parainfluenza 3 virus, HeLa cell monolayers in 250-ml, plastic, tissue culture flasks (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) were each inoculated with 3 ml of an appropriate virus dilution. After incubation at 35 C for 2 h, residual inoculum was removed, and 15 ml of maintenance medium was added per flask. Cell cultures were then incubated at 35 C until a cytopathic effect was observed. The contents of each flask were then frozen (−70 C) and thawed (35 C) three times, pooled, and centrifuged at 170 × g for 5 min. The supernatant fluid was dispensed and stored as described for the influenza virus pool.

Erythrocyte suspension. Guinea pig blood obtained by cardiac puncture was mixed with an equal volume of Alsever solution and stored at 4 C. Erythrocytes were usable for up to 1 week. For each day's test, cells were washed three times in phosphate-buffered saline (PBS), pH 7.2. The final washing was carried out in a graduated centrifuge tube at the recommended conditions of sedimentation (9). Packed cells were resuspended in PBS to make a 0.4% erythrocyte suspension that was calculated by volume.

Virus hemadsorption assay. Virus dilutions prepared in saline (0.2 M NaCl) buffered by 0.01 M

phosphate (PBS), pH 7.2, were introduced in 0.2-ml volumes directly onto cover slip cell monolayers held in rotor chamber inserts (6). These were employed because they withstand the centrifugal force required to sediment virus. Rotor chamber inserts placed in a swinging-bucket SB-110 rotor were centrifuged in a model B-50 ultracentrifuge (International Equipment Co., Needham Heights, Mass.) at 10,000 rpm (9,838 to 16,155 × g, depending on the distance of the chamber insert in the arm of the rotor from the axis of rotation) for 12 min at 6 C. Residual inoculum was removed after centrifugation, the cover slip cell monolayers were placed into glass vials, and 1 ml of maintenance medium was then added to each vial. After incubation at 35 C for 20 to 24 h, cover slip cell monolayers were rinsed with PBS. A 0.4% suspension of guinea pig erythrocytes in PBS was added in a 0.5-ml volume onto each cell monolayer which was then held at 6 C for 20 min.

For enumerating cells exhibiting hemadsorption, cover slip cell monolayers were examined with a Nikon inverted microscope. With this optical system at a magnification of ×200, the number of microscopy fields contained in the area of a 15-mm diameter cover slip was 365. For each cover slip cell monolayer, 30 microscopy fields were examined. To calculate the number of hemadsorption cell units of virus per milliliter, the average number of cells showing hemadsorption per field was multipled by the number of field per cover slip, the reciprocal of the dilution of virus inoculum, and the volume factor (for conversion to milliliters).

Virus immunofluorescence assay. This procedure is based on counting fluorescent cells in infected cell monolayers that have been stained with viral antiserum conjugated with fluorescein isothiocyanate. In all aspects, the infection and incubation conditions for cell monolayers were identical to that described for the virus hemadsorption assay. The direct fluorescent-antibody method was used to demonstrate immunofluoresence of viral antigens in infected cells. Details of the staining procedure, materials, microscope equipment, and enumeration of infected cells have been described elsewhere (7).

HA titration. Test samples were assayed for HA by using the standard Microtiter system with plastic "V"-bottomed plates. In 0.05-ml amounts, test preparations were serially diluted in PBS in twofold steps. Each well then received 0.05 ml of a 0.5% chicken erythrocyte suspension, and after incubation at room temperature from 1 to 2 h, test patterns were read. The HA titer was the reciprocal of the highest dilution of test sample that showed complete agglutination of cells.

Determination of virus attachment. Attachment was measured by following the disappearance of virus from inoculum after its addition to cell monolayers. Virus inoculum at multiplicity of infection of 0.01 in a 0.2-ml volume was introduced onto cells. After designated intervals of incubation or centrifugation, residual inoculum was removed, cell cultures were immediately washed with PBS, and residual inoculum was introduced onto fresh cell monolayers to measure unattached virus. Cover slip cell monolayers exposed to initial or residual inocula were treated in the

manner described earlier for virus assay. The amount of virus that was attached to cells at a given time was expressed as a percentage of the virus input. The latter was the sum of the amounts of attached and unattached virus.

Determination of virus penetration. Virus penetration into cells was measured by the insensitivity of attached virus to antiserum. After inoculum was attached to cells by centrifugation (6 C), cell cultures were washed with PBS, overlaid at designated intervals of incubation with 0.5 ml of a prewarmed 1:15 dilution of virus antiserum, and then incubated at 35 C for 2 h. The antiserum was removed and replaced with maintenance medium, and incubation of cell cultures was continued for an additional 20 h. The quantity of virus that penetrated into cells at a given time was expressed as a percentage of the input virus.

RESULTS

Standardization of the assay. The efficiency of procedures employed to initiate virus infection of cells by using hemadsorption as the monitor of cell infection was investigated. The rate of influenza and parainfluenza 3 virus attachment was determined during stationary incubation (35 C) and during centrifugation (6 C). Aided by centrifugal force, approximately 98% of the influenza virus inoculum was attached within 10 min; after stationary incubation for 2.5 h, only 37% of the inoculum was attached (Table 1).

The rate of penetration of influenza virus into cells at 35 C was followed by determining the insensitivity of attached virus to antiviral serum at designated times. Results show that penetration proceeded at a linear rate and was complete within 15 min (Fig. 1). The findings on influenza virus attachment and penetration were comparable to those noted with parainfluenza 3 virus.

The incubation period is defined as the time

TABLE 1. *Centrifugation versus stationary incubation for attachment of influenza (PR8) virus onto clone 1-5C-4 cell monolayers*

Min	Inoculum attached (%)	
	Centrifugation[a]	Stationary (35 C) incubation
5	83.7	ND[b]
10	98.9	ND
15	97.0	ND
30	ND	13.0
60	ND	19.6
90	ND	27.8
120	ND	30.0
150	ND	37.1

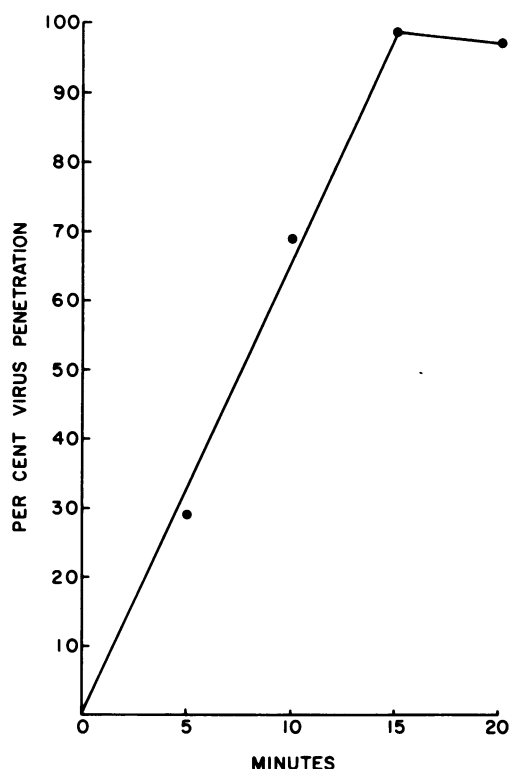[a] Conditions: 16,155 × g; 6 C.
[b] Not determined.

FIG. 1. *Rate of penetration of influence (PR8) virus into clone 1-5C-4 cells at 35 C.*

interval between virus inoculation of cell monolayers and the development of hemadsorption capability by maximal numbers of infected cells. This period was established from sequential sampling of cell monolayers inoculated with either influenza or parainfluenza 3 viruses at a multiplicity of infection of 0.01 and incubated at 35 C. Cell monolayers were then periodically tested for hemadsorption. At the same time

interval, additional infected-cell monolayers were frozen (-70 C) and thawed (35 C) twice and tested for hemagglutinin and the production of infectious virus. Hemadsorption was detected within 7 h after virus inoculation of cell monolayers (Table 2). The general pattern of erythrocyte adherence to infected cells was in the form of rosettes or clusters (14). The maximal number of cells exhibiting hemadsorption occurred between 20 and 24 h of the primary cycle of infection. The production of infectious virus and hemagglutinin was not detected earlier than 48 h.

The susceptibility of five established cell lines to infection by influenza and parainfluenza 3 viruses was determined by the virus hemadsorption assay. Virus infectivity for each cell line was carried out in triplicate. Results indicate that clone 1-5C-4, LLC-MK$_2$, and BHK-21/C13 cell lines were equally susceptible to infection by influenza virus; L-929 and HeLa cell lines were less susceptible. The following cell lines were susceptible to parainfluenza 3 virus infection (in order of decreasing susceptibility): BHK-21/C13, LLC-MK$_2$, 1-5C-4, HeLa, and L-929. The BHK-21/C13 cell line was not suitable for the hemadsorption assay of virus. With this cell line, cells infected by either influenza or parainfluenza 3 viruses exhibited a "streamer effect" (14) in the presence of guinea pig erythrocytes, making it difficult to count accurately individual cells exhibiting hemadsorption.

To determine the optimal incubation conditions for adsorption of erythrocytes to infected cells, the reaction was observed at varied temperatures (6 and 22 C) and time intervals (5 to 30 min) in cell monolayers infected with either influenza or parainfluenza 3 viruses. Results (Table 3) show that maximal hemadsorption occurred in influenza or parainfluenza 3 virus-

TABLE 2. *Sequence of viral antigen formation in clone 1-5C-4 cells infected with myxoviruses at a multiplicity of infection of 0.01*

Time after infection (h)	Influenza (PR8)			Parainfluenza 3		
	Hemadsorption[a]	Hemagglutin[b]	Infectious virus[c]	Hemadsorption	Hemagglutinin	Infectious virus
4	0	0	0	0	0	0
7	1.5×10^1	0	0	4.7×10^1	0	0
12	1.6×10^3	0	0	5.0×10^2	0	0
16	2.2×10^3	0	0	4.3×10^3	0	0
20	3.0×10^3	0	0	7.5×10^3	0	0
24	2.6×10^3	0	0	1.6×10^4	0	0
48	3.2×10^3	ND[d]	1.3×10^3	7.0×10^4	32	1.3×10^4

[a] Number of cells exhibiting hemadsorption on a 15-mm cover slip cell culture per 0.2 ml of inoculum.

[b] Reciprocal of highest dilution of 0.2-ml test sample showing complete hemagglutination.

[c] Number of cells with fluorescent antigen on a 15-mm cover slip cell culture per 0.2 ml of inoculum.

[d] Not determined.

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TABLE 3. *Hemadsorption of guinea pig erythrocytes to virus-infected clone 1-5C-4 cell monolayers at varied conditions*

Incubation with erythrocytes (min)	Influenza (PR8) HCU[a] per ml		Parainfluenza 3 HCU per ml	
	6 C	22 C	6 C	22 C
5	4.7×10^5	7.3×10^5	4.2×10^8	3.6×10^8
10	1.2×10^6	1.2×10^6	8.4×10^8	8.9×10^8
20	3.1×10^6	2.9×10^6	1.4×10^9	1.4×10^9
30	2.9×10^6	2.9×10^6	1.4×10^9	1.3×10^9

[a] Hemadsorption cell units.

infected cell monolayers within 20 min at either 6 or 22 C. Specific attachment of erythrocytes to virus-infected cells has been reported to be independent of temperature within the range of 0 to 37 C (12).

Quantitative evaluation of the assay. A linear relationship was obtained between the number of cells manifesting hemadsorption and the relative concentration of influenza virus throughout the inoculum range of 1.5 $\log_{10}$ units (Fig. 2). These data suggest that each hemadsorption cell unit was the consequence of infection by a single virus particle or aggregate not divisible by dilution.

To estimate the precision of the hemadsorption assay for influenza and parainfluenza 3 viruses, 13 determinations were performed with each virus by the prescribed manner. The number of HCU of influenza virus per ml of inoculum ranged from 1.2×10^7 to 1.7×10^7, with a mean of 1.4×10^7 and standard deviation (SD) of ± 0.15. Expressed as a percentage, the SD was 10.7% of the mean. The number of hemadsorption cell units of parainfluenza 3 virus per milliliter of inoculum ranged from 5.0 $\times 10^8$ to 5.9×10^8, with a mean of 5.3×10^8 and SD of ± 0.29. The SD was 5.4% of the mean.

The reproducibility of the assay with the use of twofold dilutions of virus inoculum in each of the six determinations is shown in Table 4. The results attest to the reproducibility of the assay at the virus concentration levels employed.

The mode of distribution of cells exhibiting hemadsorption on a cover slip monolayer was determined by examining 145 random microscopy fields. The frequencies of fields containing cells showing hemadsorption correspond closely to the expected frequencies (Fig. 3). The chi-square test of goodness-of-fit of the observed data to the theoretical Poisson distribution showed no significant deviation (*P*-0.95 with 6 df). Cells manifesting hemadsorption were randomly distributed in cell monolayers.

The sensitivity of the hemadsorption and immunofluorescence assays for influenza and

parainfluenza 3 viruses was compared. Although these procedures involve different indicator systems of cell infection, they are both based on infective cell counting. For both assays, inoculation and incubation of infected clone 1-5C-4 cell monolayers were performed in a similar manner. Results in Table 5 show that the immunofluorescence assay for both viruses was approximately twice as sensitive as the
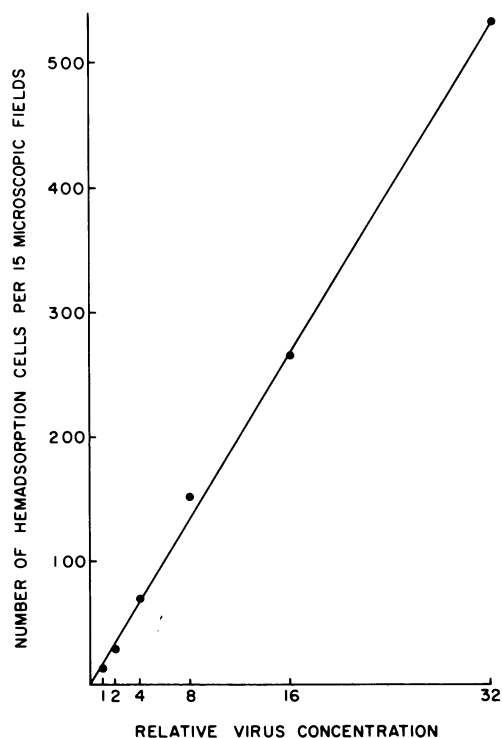

FIG. 2. *Linear function between the number of cells exhibiting hemadsorption and concentration of influenza (PR8) virus in inoculum.*

TABLE 4. *Reproducibility of hemadsorption assay for influenza (PR8) virus*

Assay no.	Dilution of virus inoculum				HCU[a]/ml ($\times 10^7$)
	1:400	1:800	1:1,600	1:3,200	
1	17.3[b]	9.0	4.2	2.1	1.25
2	17.6	8.4	4.5	2.2	1.27
3	18.3	8.5	4.6	2.0	1.26
4	17.3	9.0	4.4	2.0	1.25
5	18.6	8.3	4.4	2.0	1.25
6	17.6	8.8	4.3	2.1	1.25
Mean	17.7	8.6	4.4	2.1	1.25

[a] Hemadsorption cell units.

[b] Average number of cells exhibiting hemadsorption per 30 microscopy fields with 0.2 ml of inoculum.
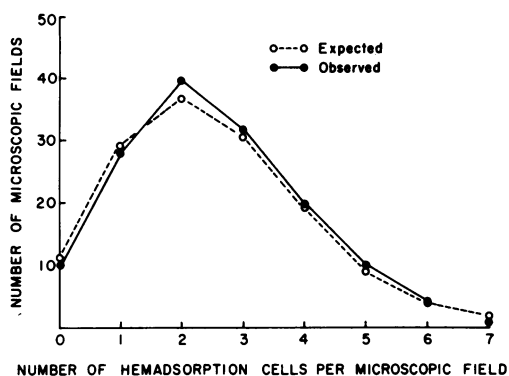
FIG. 3. *Frequency of distribution of cells exhibiting hemadsorption on 1-5C-4 cell monolayer inoculated with influenza (PR8) virus.*

TABLE 5. *Comparison of infective cell counting assays for influenza (PR8) and parainfluenza 3 viruses*

Determination	Influenza virus		Parainfluenza virus	
	Immuno-fluorescence ($\times 10^7$ CIU^a/ml)	Hemadsorption ($\times 10^7$ HCU^b/ml)	Immuno-fluorescence ($\times 10^8$ CIU/ml)	Hemadsorption ($\times 10^8$ HCU/ml)
Assay no.				
1	3.5	1.5	3.8	1.6
2	4.2	1.8	4.4	1.8
3	3.1	1.8	4.4	1.8
4	3.0	1.9	4.6	1.8
Mean	3.4	1.7	4.3	1.7
SD^c	±0.54	±0.18	±0.35	±0.01
SE^d	±0.27	±0.09	±0.17	±0.005

^{a} Cell-infecting units. Cell monolayers were infected with influenza or parainfluenza viruses at a multiplicity of 0.01 in 0.2 ml of inoculum. After incubation at 35 C for 22 h, cell monolayers were stained with fluorescein-conjugated viral antiserum.

^{b} Hemadsorption cell units. Cell monolayers were infected with influenza or parainfluenza viruses at a multiplicity of 0.01 to 0.2 ml of inoculum. After incubation at 35 C for 22 h, 0.4% guines pig erythrocytes were introduced onto cell monolayers.

^{c} Standard deviation.

^{d} Standard error of mean.

hemadsorption assay. The latter, however, exhibited slightly less variability than the former.

DISCUSSION

The feasibility of employing an assay for myxoviruses, i.e., influenza and parainfluenza 3, based on the enumeration of individual infected cells manifesting hemadsorption was established by the studies described here. Hemadsorption was detected approximately 7 h after virus inoculation of cell monolayers and was comparable in time to that observed previously (8). The maximal number of cells exhibiting hemadsorption occurred between 20 and 24 h of the primary cycle of infection. By using hemadsorption as the indicator of cell infection, resultant rates of attachment and penetration of influenza virus were similar to that attained by other means (7). The hemadsorption phenomenon appeared earlier than the production of hemagglutinins and the production of infectious virus demonstrable by immunofluorescence staining. Hemadsorption by virus-infected cells has been noted in the absence of detectable virus filaments and infectious virus particles (2, 8, 10). This suggests that the phenomenon may be attributable to an immunological structure alteration of the cell surface (10) or, more specifically, the presence of viral envelope proteins on the cell surface in the form of a "precursor" to the projections seen on virions (2).

The use of primary monkey kidney cell cultures or monkey continuous cell lines for virus assays based on hemadsorption involves serious disadvantages. A specific reaction between erythrocytes and infected cells may occur, due to the presence of myxoviruses of simian origin that occur naturally in these cell cultures (11). In addition, nonspecific tissue hemadsorption (not only common to monkey kidney cells, but which may also be manifested in kidney cell strains or lines derived from other animal species) may result (4). Although the established cell lines, clone 1-5C-4 and LLC-MK$_2$ (rhesus monkey kidney), appeared to be equally susceptible to myxovirus infection, in view of the above circumstances clone 1-5C-4 cells were preferred as the cell line for the myxovirus hemadsorption assay.

Quantitative evaluation of the assay showed that it was highly precise, reproducible, and almost equivalent in sensitivity to virus assays based on immunofluorescent cell counting (5). Limited trials performed to compare the sensitivity of the hemadsorption assay and the hemagglutination test for assessment of myxoviruses confirmed previous findings of the superiority of the former (13). In assaying influenza virus, we found that the hemadsorption assay was more than 3.0 log$_{10}$ units higher in sensitivity than the hemagglutination test.

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